# Mitochondrial DNA ligase in *Crithidia fasciculata*

**Krishna Murari Sinha, Jane C. Hines, Nicholas Downey, and Dan S. Ray***

Molecular Biology Institute and Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, CA 90095

Edited by Robert Lehman, Stanford University School of Medicine, Stanford, CA, and approved January 15, 2004 (received for review September 5, 2003)

**Kinetoplast DNA (kDNA), the form of mitochondrial DNA in trypanosomatids, consists of thousands of interlocked circular DNAs organized into a compact disk structure. A type II DNA topoisomerase, a DNA polymerase β, and a structure-specific endonuclease have been localized to antipodal sites flanking the kDNA disk along with nascent DNA minicircles. We have cloned a gene (*LIG k*) encoding a mitochondrial DNA ligase in the trypanosomatid *Crithidia fasciculata*, and we show that an epitope-tagged form of the ligase colocalizes with the other replication proteins at the antipodal sites and also at the two faces of the kDNA disk. DNA LIG k becomes adenylated in reactions with ATP, and the adenylate moiety is removed by incubation with pyrophosphate or nicked DNA. The ligase interacts physically with the β polymerase and is proposed to be involved in the repair of gaps in the newly synthesized minicircles. In yeast and mammals, a single gene encodes both nuclear and mitochondrial forms of DNA ligase. The LIG K protein sequence has low similarity to mitochondrial DNA ligases in other eukaryotes and is distinct from the *C. fasciculata* nuclear DNA ligase (LIG I).**

The mitochondrial DNA of trypanosomatid protozoa (kinetoplast DNA, or kDNA) consists of two forms of topologically interlocked circular DNAs, minicircles, and maxicircles (for recent reviews of kDNA structure and replication, see refs. 1–3). The kDNA of the trypanosomatid *Crithidia fasciculata* contains 5,000 minicircles of 2.5 kb each and 20–30 maxicircles of 37 kb each. The maxicircles encode genes for mitochondrial proteins and ribosomal RNAs. Minicircles encode small guide RNAs that serve as templates for RNA editing of maxicircle transcripts (4). *In vivo*, the kDNA exists as a condensed disk structure 1 μm in diameter and ≈0.4 μm thick. Each minicircle in the kDNA disk is linked to three other minicircles on average and is relaxed, unlike most other circular DNAs in nature, which are negatively supercoiled (5). Electron microscopic observation of sections through the kDNA disk shows fibers aligned parallel to the axis of the disk. This observation together with the thickness of the disk corresponding to approximately one half of the minicircle circumference (6) suggests that individual minicircles may have a topology like that of a rubber band stretched taut from opposite sides of the circle (7). Maxicircles appear to be present throughout the kDNA and exist as a catenated network within the overall network (8). The kDNA isolated from nonreplicating *C. fasciculata* is a planar elliptical network of interlocked circles with dimensions of ≈10 by 15 μm.

The kDNA disk is always located at the base of the flagellum, with the flagellum perpendicular to the face of the disk. Recent studies (9) have shown that in trypanosomes the kDNA disk is connected physically to the flagellar basal bodies and is segregated by them at cell division. The attachment was found to involve three components that penetrate the inner and outer mitochondrial membranes to link the kDNA physically to the basal bodies.

This DNA structure and its mechanism of replication have been the focus of studies in several laboratories for many years. The unusual organization of the kDNA network structure adds significant complexity to the replication mechanism. Not only does each minicircle and maxicircle have to be replicated during each cell cycle (10), but the network structure must be duplicated and segregated into daughter networks so that each cell receives a progeny network.

Early studies using pulse labeling and autoradiographic techniques showed that newly synthesized DNA is present at antipodal sites on the kDNA network after short-pulse labeling and spreads around the perimeter of the network with longer pulse lengths (11, 12). It was found subsequently (13) that minicircles are replicated free of the network and then reattached at the antipodal sites. The finding of a kinetoplast-specific DNA topoisomerase II located at antipodal sites flanking the kDNA disk provided a biochemical basis for the rejoining of newly replicated minicircles at these sites (14). Recent experiments with *Trypanosoma brucei* using the RNA-interference technique have provided strong support for the involvement of the homologous *T. brucei* topoisomerase II in rejoining newly replicated minicircles to the kDNA network (15).

In *C. fasciculata*, the minicircles are replicated by a unidirectional theta-type replication mechanism (16–20). One strand of the minicircle is synthesized continuously from one or the other of two replication origins located 180° apart on the minicircle, and the other strand is synthesized discontinuously. The newly replicated minicircles still contain nicks and gaps at the time of reattachment to the network perimeter. Each daughter minicircle containing the new continuously synthesized strand contains a single discontinuity at one or the other of the replication origins but not at both (19). In some molecules, the discontinuity is a nick in the phosphodiester backbone, whereas in some of these daughter molecules the discontinuity is a gap of a few nucleotides with the remnants of an RNA primer on the 5′ terminus of the gap (19, 21). The daughter minicircle containing the discontinuously synthesized strand contains multiple nicks and gaps in the newly synthesized strand. In this case, single discontinuities within each of the two replication origins are resistant to covalent closure, whereas other discontinuities within this lagging strand become closed. Remarkably, all newly synthesized minicircles remain nicked and gapped until every minicircle has been duplicated (22).

The reattachment of newly synthesized minicircles around the periphery of the kDNA network in *C. fasciculata* results in a peripheral zone of gapped minicircles and a central zone of covalently closed unreplicated minicircles. As replication proceeds, the peripheral zone enlarges while the central zone shrinks (22, 23). After all minicircles have been replicated, the network has a doubled content of minicircles, all of which still contain discontinuities. Before division of the network to yield two daughter networks, all minicircles in the double-size network become covalently closed (22). These results raise the following questions regarding the regulation of kDNA replication. (*i*) What is the basis for the differential sealing of discon-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: HA, hemagglutinin; kDNA, kinetoplast DNA.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY380335).

See Commentary on page 4333.

*To whom correspondence should be addressed at: 301A Paul D. Boyer Hall, 611 Charles Young Drive East, University of California, Los Angeles, CA 90095-1570. E-mail: danray@ucla.edu.

tinuities in the lagging strand of the minicircle? (*ii*) What is the basis for maintaining discontinuities at the replication origins until all minicircles have been duplicated? (*iii*) What signals the covalent closure of discontinuities in the entire minicircle population before division of the network?

To address these and related questions concerning the replication mechanisms of kDNA, we and other laboratories have sought to identify and characterize kinetoplast-specific replication proteins. Surprisingly, the replication proteins identified thus far show unexpected variations in their temporal and spatial localizations. After the initial discovery of the antipodal localization of the kinetoplast type II DNA topoisomerase (14), a $\beta$-type DNA polymerase (24), and a structure-specific endonuclease (SSE1) with RNase H activity (25) were found to localize at the antipodal sites along with nascent minicircles. SSE1 and polymerase $\beta$ immunofluorescence are observed at the antipodal sites during kDNA replication, but they disappear shortly after S phase (25, 26). Topoisomerase II immunofluorescence is slightly different, showing strong fluorescence at the antipodal sites during S phase and diminishing, but not disappearing completely, during cytokinesis. By using purified recombinant proteins and a model substrate, SSE1 and polymerase $\beta$ were shown to be capable of RNA primer removal and gap filling to yield a ligatable substrate (27).

Unlike the above proteins, a DNA primase and a minicircle origin binding protein (the universal minicircle sequence binding protein, or UMSBP) have distinctly different localizations (28, 29). The DNA primase localizes to the two faces of the kDNA disk and not at the antipodal sites. UMSBP, however, is observed at two sites on the flagellar face of the kDNA disk distinct from the two antipodal sites. As replication proceeds, the UMSBP immunofluorescence becomes more diffuse, and the immunofluorescence is lost as cells divide.

The final sealing of discontinuities in the population of newly replicated minicircles appears to be regulated precisely and is prerequisite to the division of the double-size network to give rise to daughter networks (22, 23). We have sought to identify additional proteins involved in these terminal steps in the duplication of the kDNA network. We describe here the identification and characterization of a kinetoplast-specific DNA ligase in *C. fasciculata* and a gene encoding a similar protein from *T. brucei*.

## Materials and Methods

**Cloning the *C. fasciculata* kDNA Ligase Gene.** Degenerate primers were designed by using amino acid sequence translated from *Leishmania major* DNA sequence of a putative mitochondrial DNA ligase (AL160493). When using *C. fasciculata* genomic DNA as template, primer pairs TTCGCSTTCGACATC ATGTAY and GTGGAAGTACTTSCCGTCYTT produced a *Taq* polymerase PCR product of a predicted size (≈475 bp). Sequencing of the PCR product confirmed it to be a homolog of the *L. major* gene. This PCR product was used to screen a λGEM-11 (Promega) *C. fasciculata* genomic DNA library (30). A 3.6-kb MluI fragment, which contained the PCR product, was subcloned from a positive phage isolate into the MluI site of pGEM7 (Promega), creating the plasmid pJH42. Sequence analysis of the 3.6-kb fragment showed that it contained the entire DNA ligase coding sequence.

**Expression of Hemagglutinin (HA)-Tagged DNA Ligase in *C. fasciculata*.** The plasmid pGEM7Zf(+) (Promega) was engineered to remove its Bsp120I site, followed by cloning of the 3.6-kb MluI fragment of pJH42 into the MluI site of pGEM7Zf(+). Six copies of the influenza HA epitope were then cloned into an in-frame Bsp120I restriction site, 24 bp upstream of the termination codon, to yield the plasmid pMS50.3. A neomycin drug-resistance cassette (31) was cloned downstream of the $HA_6$-tagged ligase gene in pMS50.3 by swapping its *Xba*I fragment with the *Xba*I fragment of pX.2KO (32) to create the expression construct pMS50.4. Wild-type *C. fasciculata* cells were transformed with pMS50.4 and selected on nutrient plates containing 50 $\mu$g/ml G418, as described (32).

**Immunolocalization and Confocal Microscopy.** Immunofluorescent localization of HA-tagged ligase was performed essentially as described (33). Briefly, cells at $2 \times 10^7$ per ml were harvested, resuspended in PBS, spotted onto poly-L-lysine-coated slides, and allowed to adhere for 30 min in a humid chamber. The cells were then fixed in 4% paraformaldehyde in PBS for 5 min. Fixation was stopped by two 5-min washes in 0.1 M glycine in PBS, followed by incubation for 5 min in 0.1% Triton X-100 in PBS. The slides were kept in methanol at −20°C overnight, rehydrated by washing three times in PBS, and then blocked for 30 min at 37°C in 20% goat serum in PBST (PBS/0.05% Tween-20). The cells were then incubated at 37°C for 45 min with a mixture of mouse 12CA5 mAb (Babco, Richmond, CA) at a 1:100 dilution to detect the HA-tagged ligase and rabbit anti-polymerase $\beta$ polyclonal Ab at a 1:500 dilution to detect DNA polymerase $\beta$ (26). The cells were then washed three times in PBST for 5 min each and incubated with Alexa Fluor 488 goat anti-mouse IgG (2 mg/ml) at a 1:50 dilution and Alexa Fluor 568 goat anti-rabbit IgG (2 mg/ml) at a 1:400 dilution (Molecular Probes) for 30 min at 37°C. The slides were washed again three times for 5 min each in PBST and then mounted by using ProLong Antifade (Molecular Probes), containing 2 $\mu$M TO-PRO-3 iodide (Molecular Probes). All of the incubations at 37°C were done in a humid chamber. The images were captured on a Radiance 2000 confocal microscope (Bio-Rad).

**Partial Purification of HA-Tagged DNA Ligase from *C. fasciculata* Cells.** *C. fasciculata* cells containing the plasmid pMS50.4 were grown at 28°C in 1 liter of 3.7% (wt/vol) brain–heart infusion broth (Difco), supplemented with 20 $\mu$g/ml hemin and 100 $\mu$g/ml streptomycin in the presence of 50 $\mu$g/ml G418. The cells were grown in two 2.8-liter flasks to a density $7 \times 10^7$ cells per ml and then harvested by centrifuging at 4°C; the cells and cell lysate were kept at 4°C throughout all procedures. The cells were washed once in cold PBS and then disrupted by homogenization in 1.25% Triton X-100 in a Buffer A, containing 250 mM KCl, 10% glycerol, 30 mM Tris (pH 8.0), 2 mM DTT, 1 mM EDTA, 2 mM benzamidine hydrochloride, and 0.5 mM phenylmethylsulfonyl fluoride. The lysate was incubated further on ice for 30 min, followed by centrifugation for 1 h at 40,000 rpm in a Ti50 rotor (Beckman Coulter). The supernatant was collected while carefully avoiding the turbid mass floating on top and then passed through a 25-ml DE-52 column that was preequilibrated with Buffer A. The flow-through was dialyzed against Buffer B (20 mM potassium phosphate, pH 7.4/10% glycerol/2 mM DTT/0.1 mM EDTA/2 mM benzamidine hydrochloride/0.5 mM phenylmethylsulfonyl fluoride) and then centrifuged at 10,000 rpm for 10 min to remove any precipitate. The supernatant was then loaded onto a 30-ml phosphocellulose column preequilibrated with Buffer B. The column was washed with 100 ml of Buffer B, and the bound proteins were eluted with a linear gradient of 0–750 mM KCl in 150 ml of Buffer B. Fractions were analyzed for DNA ligase activity by using the adenylation assay described below. The fraction containing the highest activity of HA-tagged DNA ligase was dialyzed against Buffer A without KCl and used for adenylation and immunoprecipitation studies.

**Ligase Adenylation.** Adenylation reactions were performed to determine the DNA ligase activity of the phosphocellulose eluate (34). Reaction mixture (10 $\mu$l) contained 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 8% glycerol, 0.02% Triton X-100, 0.2 $\mu$Ci (1 Ci = 37 GBq) of [$\alpha$-$^{32}$P]ATP, and 0.5 $\mu$l of

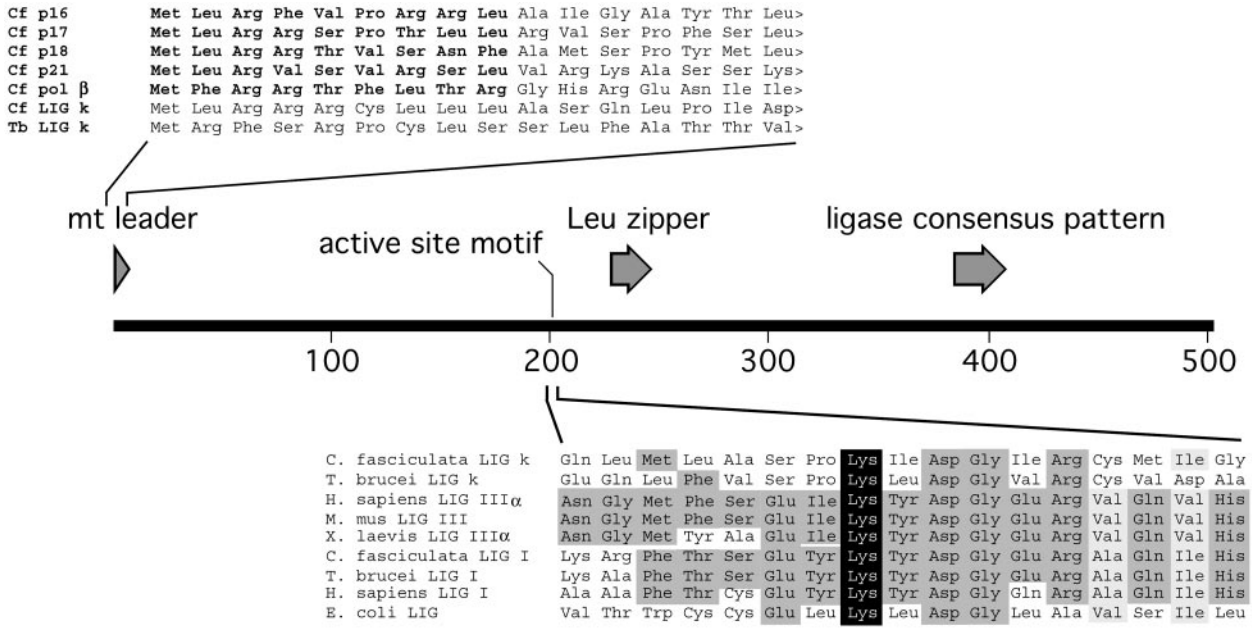


**Fig. 1.** Schematic representation of the 502-aa *C. fasciculata* mitochondrial DNA ligase protein and protein-sequence alignments. The N-terminal sequences of the *C. fasciculata* and *T. brucei* mitochondrial ligases are shown above the schematic diagram aligned with N-terminal sequences of *C. fasciculata* proteins known to have cleavable leader sequences (in bold). Sequences around the ligase active-site motif are shown below the schematic diagram aligned with those of several known mitochondrial (LIG III) and nuclear (LIG I) DNA ligases. The active-site lysine is indicated in white text on a black background. A ligase-consensus pattern (PROSITE) and a leucine-zipper sequence are indicated by arrows. GenBank accession nos. are as follows: AAB25701 (p16), AY143553 (p17), AF008943 (p18), AAC32801 (p21), AAA68599 (polymerase β), NM002311 (*Homo sapiens* LIG IIIα), MMU66058 (*Mus musculus* LIG IIIα), AF393654 (*Xenopus laevis* lig IIIα), CAA80615 (*C. fasciculata* LIG I), AAQ88427 (*C. fasciculata* LIG k), AC007863 (*T. brucei* LIG I), AAQ72485 (*T. brucei* LIG kα), AAA59518 (*H. sapiens* LIG I), and AAC75464 (*Escherichia coli* LIG).

enzyme fraction. The reaction mixture was incubated at 25°C for 15 min, stopped by adding SDS sample buffer, and boiled for 5 min. The proteins were resolved on 10% SDS/PAGE, fixed in 30% methanol and 10% acetic acid, dried, and detected by autoradiography.

**Immunoprecipitation of HA-Tagged DNA Ligase.** Adenylated proteins from the phosphocellulose eluate were immunoprecipitated with mouse 12CA5 mAb. In brief, sodium chloride and Nonidet P-40 were added to the adenylation reaction mixture to final concentrations of 100 mM and 0.5%, respectively. The reaction mixture was precleared with 50 μl of protein A-Sepharose slurry (1:1 dilution) in equilibration buffer (25 mM Tris, pH 8.0/0.5% Nonidet P-40/150 mM NaCl) by gently agitating in the cold for 30 min. The precleared reaction mixture was then incubated with Abs for 3 h in the cold, followed by addition of 50 μl of protein A-Sepharose (1:1 dilution). The reaction mixture was agitated gently for 2 h in the cold. The beads were washed three times with equilibration buffer for 5 min each. The beads were then boiled in SDS sample buffer for 5 min. Equivalent aliquots of the supernatant and immunoprecipitate were analyzed on 10% SDS/PAGE, fixed, dried, and detected by autoradiography.

**Deadenylation of DNA Ligase.** Adenylated DNA ligase was discharged with poly(dA)·oligo(dT) or DNase I-activated calf thymus DNA (34). After adenylation, 10 μl of reaction mixture was incubated separately with 10 μl of 10 mM sodium pyrophosphate, 10 μl of 250 ng/μl poly(dA)·oligo(dT), 2 μl of 2.5 μg/μl DNase I-treated calf thymus DNA, or 10 μl of 250 ng/μl poly(rA)·oligo(dT) at 25°C for 15 min. The reaction mixtures were boiled with SDS sample buffer and analyzed by 10% SDS/PAGE. The gel was fixed in 30% methanol and 10% acetic acid for 30 min, dried, and autoradiographed.

**Coimmunoprecipitation of DNA Polymerase β and DNA Ligase.** *C. fasciculata* cells transformed with pMS50.4 were grown to a density of $4 \times 10^7$ cells per ml. The cells were lysed by suspending in lysis buffer containing 25 mM Tris (pH 8.0), 100 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 20 μg/ml leupeptin, and 1 μg/ml pepstatin A at 4°C (all procedures were carried out in the cold unless stated otherwise) and incubated further on ice for 30 min. Cell lysate corresponding to $3 \times 10^8$ cells was precleared with 50 μl of protein A-Sepharose slurry (1:1 dilution) in lysis buffer by gentle agitation for 30 min. Anti-DNA polymerase β was then added to the supernatant and incubated with gentle agitation for 5 h. We added 50 μl of protein A-Sepharose slurry (1:1 dilution), and incubation was continued for 2 h. Protein A-Sepharose beads were washed two times for 5 min each in 500 μl of lysis buffer and then boiled for 5 min in SDS sample buffer without β-mercaptoethanol. The proteins were separated on 10% SDS/PAGE and then processed for Western blotting, as described (30). The membrane was probed with 1:5,000 anti-HA 12CA5 mAb.

## Results

**Trypanosomatid DNA Ligases.** A search of the *L. major* genome database revealed a putative DNA ligase gene with an N-terminal sequence similar to cleavable mitochondrial leader sequences identified in *C. fasciculata* (Fig. 1). The corresponding *C. fasciculata* gene was cloned from a genomic library by using degenerate primers based on the *Leishmania* sequence. The translated *C. fasciculata* sequence predicts a protein of 56.6 kDa with a pI of 9.66. The predicted sequence is highly divergent from previously described DNA ligases, but it contains a ligase-consensus pattern and the ligase active-site motif -K-DG-R- (35), which is essential for formation of an enzyme–adenylate reaction intermediate (Fig. 1). The *C. fasciculata* DNA ligase

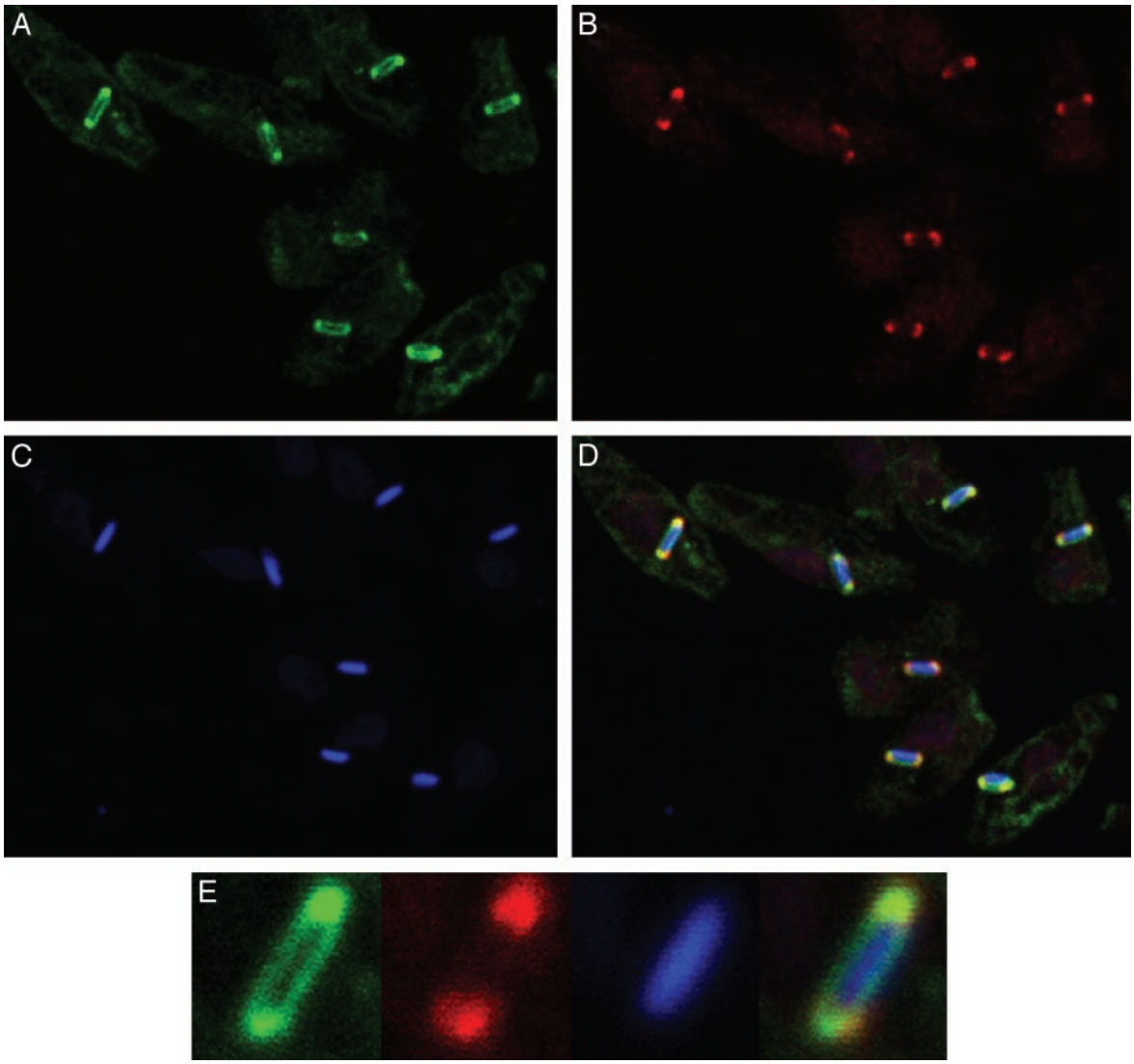


**Fig. 2.** Immunolocalization of the *C. fasciculata* DNA ligase. *C. fasciculata* cells transformed with pMS50.4 expressing the HA epitope-tagged DNA ligase were immunostained with mouse mAbs against the HA epitope and with rabbit polyclonal Abs against the mitochondrial DNA polymerase $\beta$. (*A*) Immunofluorescence of LIG k. (*B*) Immunofluorescence of polymerase $\beta$. (*C*) DNA fluorescent stain. (*D*) Merged images. (*E*) Higher magnification of fluorescence from a single kinetoplast.

active-site sequence shown in Fig. 1 differs from that of the *L. major* ligase sequence (data not shown) only at the first and third positions in the sequence. The *C. fasciculata* ligase gene is clearly distinct from the *C. fasciculata* nuclear DNA ligase I described (36) and also is unrelated to the mitochondrial DNA ligases (LIG III) of higher eukaryotes. However, the predicted N-terminal sequence of the kinetoplast ligase is consistent with the 9-aa cleavable sequences of several other kinetoplast-targeted proteins in *C. fasciculata* (37). Interestingly, the *C. fasciculata* nuclear *LIG I* gene is highly similar to *LIG I* genes of higher eukaryotes.

To investigate the possible conservation of the kDNA ligase active-site motif in a more distantly related kinetoplastid, we have identified a putative *T. brucei* kDNA ligase by using the translated protein sequence of the putative *L. major* kDNA ligase gene in a BLAST search of the *T. brucei* genome database (available at www.ebi.ac.uk/parasites/parasite-genome.html). The resulting sequences were used in a BLAST search of the same database until a contiguous sequence covered a complete ORF. When translated, this sequence appeared from PROSITE (available at http://us.expasy.org/tools/scanprosite) analysis to have a kinetoplast targeting sequence and DNA ligase motif (Fig. 1). PCR primers were designed from the ORF sequence and used to amplify the gene from *T. brucei* strain 29–13 genomic DNA. The PCR product was cloned into the pCR II-TOPO vector (Invitrogen), and both strands were sequenced (GenBank accession no. AY346452). The resulting sequence shows that the sequences around the active-site lysine are highly conserved in these kinetoplastid species (Fig. 1).

**Kinetoplast Localization of the DNA Ligase.** An epitope-tagged form of the *C. fasciculata* DNA ligase was expressed from a plasmid construct in *C. fasciculata* to determine the intracellular localization of the ligase protein. Fig. 2 shows the immunolocalization of the epitope-tagged ligase and the kDNA polymerase $\beta$. The DNA ligase localizes primarily to antipodal sites flanking the kinetoplast disk and also, to a lesser extent, to the two faces of the disk. The antipodal localization of the ligase overlaps that of the polymerase $\beta$ where the kinetoplast topoisomerase II and SSE1 localize also,

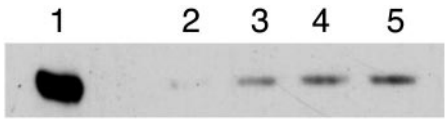


**Fig. 3.** Epitope-tagged mitochondrial DNA ligase coimmunoprecipitates with the mitochondrial DNA polymerase $\beta$. Lane 1 shows cell lysate corresponding to $1.5 \times 10^6$ cells. Cell lysate corresponding to $3 \times 10^8$ cells was immunoprecipitated with 0, 0.5, 1.0, and 2.0 $\mu$l of Abs against DNA polymerase $\beta$ in lanes 2–5, respectively. The blot was probed with 12CA5 mAbs against the HA-epitope tag.

bringing the number of DNA replication proteins at the antipodal sites to a total of four.

**Interaction with the $\beta$ Polymerase.** The presence of many replication proteins at the antipodal sites suggests that these proteins may interact physically with one another. Because the polymerase $\beta$ has been implicated in the repair of discontinuities in nascent minicircles, an interaction of the DNA ligase with the $\beta$ polymerase might facilitate this repair. Evidence of such interaction was obtained by immunoprecipitation of polymerase $\beta$ from extracts of cells expressing the epitope-tagged DNA ligase. Western blotting of the precipitates with the 12CA5 mAbs shows that the ligase coprecipitates with the polymerase $\beta$ (Fig. 3). Similarly, bovine nuclear polymerase $\beta$ has been found to interact with a nuclear DNA ligase (38).

**Adenylation of the Kinetoplast Ligase.** DNA and RNA ligases catalyze the formation of enzyme–AMP complexes as intermediates in the ligation reaction, and this reaction can be used in the detection and purification of ligases. We have fractionated extracts of *C. fasciculata* expressing the epitope-tagged ligase by phosphocellulose column chromatography (data not shown) and have detected multiple proteins that become adenylated in reactions with [$\alpha$-$^{32}$P] ATP (Fig. 4*A*, lane 1). Immunoprecipitation of this fraction with 12CA5 Abs results in the precipitation of the 68-kDa protein (Fig. 4*A*, lanes 7 and 8) and the loss of that protein from the supernatant (Fig. 4*A*, lanes 3 and 4). Mock incubation (Fig. 4*A*, lane 2) or incubation with a nonspecific Ab (Fig. 4*A*, lane 5) failed to precipitate any of the adenylatable proteins.

A characteristic feature of DNA ligases is the ability to remove the adenylate moiety from the enzyme–AMP complex by either the reversal of the reaction by incubation with pyrophosphate in the absence of a nucleic acid substrate or by reaction with a nicked DNA substrate to transfer the adenylyl group to the 5′-P terminus of the DNA to generate DNA-adenylate. Of the four major species adenylated in the phosphocellulose fraction, the $^{32}$P label is lost from all of the proteins on incubation with pyrophosphate (Fig. 4*B*, lane 3) and is reduced significantly in the 68- and 56-kDa species on incubation with poly(dA)·oligo(dT) (Fig. 4*B*, lane 4) or DNase I-treated calf thymus DNA (Fig. 4*B*, lane 5). Incubation with poly(rA)·oligo(dT) had no effect on any of the adenylated proteins. Of the four major adenylated proteins, the 68- and 56-kDa species appear, based on their ability to be deadenylated by nicked DNA, to be DNA ligases, whereas the 48- and 43-kDa species are close in size to kinetoplast RNA ligases identified in *Leishmania tarentolae* (GenBank accession nos. AY148476 and AY148475). Immunoprecipitation of the 68-kDa species indicates that it corresponds to the $HA_6$ epitope-tagged protein encoded by the cloned ligase gene. The 56-kDa species likely represents the mature form of the endogenous kDNA ligase, which is predicted to have a molecular mass of 55.5 kDa after removal of a 9-aa presequence. Taken together, these results

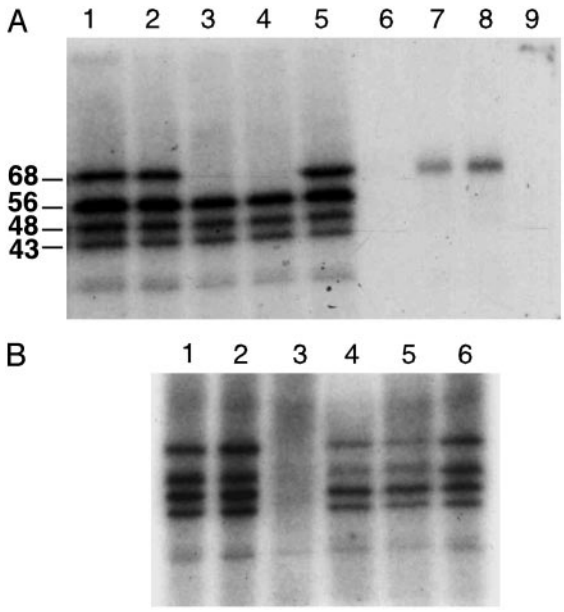


**Fig. 4.** (*A*) Immunoprecipitation of adenylated DNA ligase. Lane 1 shows adenylation of phosphocellulose purified proteins. Lanes 2–5, show supernatants from immunoprecipitation reactions incubated with 0, 0.1, and 0.5 $\mu$l of 12CA5 mAbs or 1 $\mu$l of anti-CSBPB (44), respectively. Lanes 6–9 show immunoprecipitates from the reactions run in lanes 2–5. (*B*) Deadenylation of HA-tagged DNA ligase. The adenylated proteins (lane 1) were mock incubated (lane 2) or incubated with sodium pyrophosphate (lane 3), poly(dA)·oligo(dT) (lane 4), DNase I-treated calf thymus DNA (lane 5), or poly(rA)·oligo(dT) (lanes 6).

identify the cloned gene as that of a DNA ligase, consistent with the identification based on sequence analysis.

## Discussion

It has been known for a long time that discontinuities at the replication origins of kDNA minicircles are resistant to closure compared with those of the discontinuously synthesized strand of the minicircle (19, 39). It has been suggested that these discontinuities may provide a means for distinguishing replicated minicircles from the covalently closed minicircles that have not yet been replicated (7). Identification of the enzymatic machinery involved in the repair of nicks and gaps in minicircle DNA is an important step in understanding this form of regulation. The *C. fasciculata* kDNA ligase identified here is likely to be involved in the joining of Okazaki fragments in nascent minicircles. In light of the multiplicity of DNA polymerases in the kinetoplasts of *T. brucei* (40), it would not be surprising to find additional DNA ligases in kinetoplasts. The additional DNA ligase observed here in the adenylation reactions has an apparent molecular mass that is much less than that of the nuclear ligase I (84.2 kDa) but close to that of the endogenous form of LIG k. While prepararing this article, we identified an additional putative DNA ligase gene closely linked to the *L. major* gene that led to our cloning of the *C. fasciculata* kDNA ligase, and closely related linked genes have been identified also in the *T. brucei* database (N.D. and D.S.R., unpublished data).

The *C. fasciculata* kDNA ligase, which we term LIG k, is encoded by a gene that is separate from that of the nuclear DNA ligase (*LIG I*), whereas in yeast (41) and humans (42), a single gene encodes both nuclear and mitochondrial forms of DNA ligase. Four distinct classes of DNA ligase (LIG I–IV) have been identified in mammalian cells, and LIG I ligates Okazaki fragments in the nucleus. The *Crithidia* LIG I has been localized to the nucleus and is 48% similar (35% identical) to human LIG I.

The LIG k protein, on the other hand, shows very little relationship to the human mitochondrial DNA ligase (20% similarity, 11% identity), which is encoded by *LIG III* and has a molecular mass of ≈100 kDa (43). *LIG k* is predicted to encode a preprotein of 56.6 kDa, and cleavage of the predicted 9-aa leader sequence would result in a protein of 55.5 kDa. The lack of significant relationship of the LIG k to mitochondrial ligases in other eukaryotes is reminiscent of the situation with kDNA polymerases. Paul Englund's laboratory (ref. 40 and personal communication) has identified six kDNA polymerases in *T. brucei*, yet none of them are similar to the typical $\gamma$ polymerases of other eukaryotes. In both cases, the kinetoplastid proteins share more similarity with prokaryotic sequences than with eukaryotic sequences.

The localization of the *C. fasciculata* kDNA ligase both to the antipodal sites and to the faces of the kinetoplast disk raises some interesting possibilities regarding the possible role(s) of this DNA ligase in minicircle replication. Localization to the antipodal sites and association with a repair-type DNA polymerase (pol $\beta$) suggest a role for the ligase in joining Okazaki fragments on the discontinuously synthesized strand of minicircles. However, the newly replicated minicircles still contain discontinuities at the origins of replication at the time of their reattachment to the kDNA network and are repaired only after their dispersal throughout the network and after all minicircles have been replicated. The additional presence of this DNA ligase at the two faces of the kinetoplast disk suggests that the same DNA ligase might also be involved in the final repair of the discontinuities at the replication origins. Although the precise orientation of individual minicircles in the kDNA disk is unknown, the model in which the minicircles are stretched taut from opposite sides might be considered, particularly if the two replication origins that are located 180° apart on the minicircle map are located at the two ends of the taut DNA circle. This organization would place the nicked and/or gapped replication origins at the opposite faces of the kDNA disk, which might explain the localization of the DNA ligase at the two faces of the disk.

We thank Paul Englund for kindly providing antiserum against *C. fasciculata* polymerase $\beta$ and Utpal Banerjee and Cory Evans for assistance with confocal microscopy. This work was supported by National Institutes of Health Grant GM53254.

1. Shapiro, T. A. & Englund, P. T. (1995) in *Annu. Rev. Microbiol.*, **49,** 117–143.
2. Klingbeil, M. M., Drew, M. E., Liu, Y., Morris, J. C., Motyka, S. A., Saxowsky, T. T., Wang, Z. & Englund, P. T. (2001) *Protist* **152,** 255–262.
3. Morris, J. C., Drew, M. E., Klingbeil, M. M., Motyka, S. A., Saxowsky, T. T., Wang, Z. & Englund, P. T. (2001) *Int. J. Parasitol.* **31,** 453–458.
4. Sturm, N. R. & Simpson, L. (1990) *Cell* **61,** 879–884.
5. Chen, J., Rauch, C. A., White, J. H., Englund, P. T. & Cozzarelli, N. R. (1995) *Cell* **80,** 61–69.
6. Simpson, L. (1972) *Int. Rev. Cytol.* **32,** 139–207.
7. Englund, P. T., Ferguson, M., Guilbride, D. L., Johnson, C. E., Li, C., Perez-Morga, D., Rocco, L. J. & Torri, A. F. (1995) in *Molecular Approaches to Parasitology*, eds. Boothroyd, J. C. & Komuniecki, R. (Wiley–Liss, New York), Vol. 49, pp. 147–161.
8. Shapiro, T. A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7809–7813.
9. Ogbadoyi, E. O., Robinson, D. R. & Gull, K. (2003) *Mol. Biol. Cell* **14,** 1769–1779.
10. Hajduk, S. L., Klein, V. A. & Englund, P. T. (1984) *Cell* **36,** 483–492.
11. Perez-Morga, D. L. & Englund, P. T. (1993) *Cell* **74,** 703–711.
12. Simpson, A. M. & Simpson, L. (1976) *J. Protozool.* **23,** 583–587.
13. Englund, P. T. (1979) *J. Biol. Chem.* **254,** 4895–4900.
14. Melendy, T., Sheline, C. & Ray, D. S. (1988) *Cell* **55,** 1083–1088.
15. Wang, Z. & Englund, P. T. (2001) *EMBO J.* **20,** 4674–4683.
16. Kitchin, P. A., Klein, V. A., Fein, B. I. & Englund, P. T. (1984) *J. Biol. Chem.* **259,** 15532–15539.
17. Ntambi, J. M. & Englund, P. T. (1985) *J. Biol. Chem.* **260,** 5574–5579.
18. Birkenmeyer, L. & Ray, D. S. (1986) *J. Biol. Chem.* **261,** 2362–2368.
19. Birkenmeyer, L., Sugisaki, H. & Ray, D. S. (1987) *J. Biol. Chem.* **262,** 2384–2392.
20. Sheline, C., Melendy, T. & Ray, D. S. (1988) *Mol. Cell. Biol.* **9,** 169–176.
21. Ntambi, J. M., Shapiro, T. A., Ryan, K. A. & Englund, P. T. (1986) *J. Biol. Chem.* **261,** 11890–11895.
22. Perez-Morga, D. & Englund, P. T. (1993) *J. Cell Biol.* **123,** 1069–1079.
23. Guilbride, D. & Englund, P. (1998) *J. Cell Sci.* **111,** 675–679.
24. Ferguson, M., Torri, A. F., Ward, D. C. & Englund, P. T. (1992) *Cell* **70,** 621–629.
25. Engel, M. L. & Ray, D. S. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 8455–8460.
26. Johnson, C. E. & Englund, P. T. (1998) *J. Cell Biol.* **143,** 911–919.
27. Hines, J. C., Engel, M. L., Zhao, H. & Ray, D. S. (2001) *Mol. Biochem. Parasitol.* **115,** 63–67.
28. Abu-Elneel, K., Robinson, D. R., Drew, M. E., Englund, P. T. & Shlomai, J. (2001) *J. Cell Biol.* **153,** 725–734.
29. Johnson, C. E. & Englund, P. T. (1999) *Mol. Biochem. Parasitol.* **102,** 205–208.
30. Pasion, S. G., Hines, J. C., Aebersold, R. & Ray, D. S. (1992) *Mol. Biochem. Parasitol.* **50,** 57–67.
31. Cruz, A., Coburn, C. M. & Beverley, S. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7170–7174.
32. Pasion, S. G., Brown, G. W., Brown, L. M. & Ray, D. S. (1994) *J. Cell Sci.* **107,** 3515–3520.
33. Lukes, J., Hines, J. C., Evans, C. J., Avliyakulov, N. K., Prabhu, V. P., Chen, J. & Ray, D. S. (2001) *Mol. Biochem. Parasitol.* **117,** 179–186.
34. Engler, M. J. & Richardson, C. C. (1982) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), Vol. 15, pp. 3–29.
35. Tomkinson, A. E., Totty, N. F., Ginsburg, M. & Lindahl, T. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 400–404.
36. Brown, G. W. & Ray, D. S. (1992) *Nucleic Acids Res.* **20,** 3905–3910.
37. Xu, C. W., Hines, J. C., Engel, M. L., Russell, D. G. & Ray, D. S. (1996) *Mol. Cell. Biol.* **16,** 564–576.
38. Prasad, R., Singhal, R. K., Srivastava, D. K., Molina, J. T., Tomkinson, A. E. & Wilson, S. H. (1996) *J. Biol. Chem.* **271,** 16000–16007.
39. Kitchin, P. A., Klein, V. A. & Englund, P. T. (1985) *J. Biol. Chem.* **260,** 3844–3851.
40. Klingbeil, M. M., Motyka, S. A. & Englund, P. T. (2002) *Mol. Cell* **10,** 175–186.
41. Willer, M., Rainey, M., Pullen, T. & Stirling, C. J. (1999) *Curr. Biol.* **9,** 1085–1094.
42. Lakshmipathy, U. & Campbell, C. (1999) *Mol. Cell. Biol.* **19,** 3869–3876.
43. Nash, R. A., Caldecott, K. W., Barnes, D. E. & Lindahl, T. (1997) *Biochemistry* **36,** 5207–5211.
44. Mahmood, R., Mittra, B., Hines, J. C. & Ray, D. S. (2001) *Mol. Cell. Biol.* **21,** 4453–4459.